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The impact of ascidian biofouling on the farmed Mediterranean mussel *Mytilus galloprovincialis* physiology and welfare, revealed by stress biomarkers

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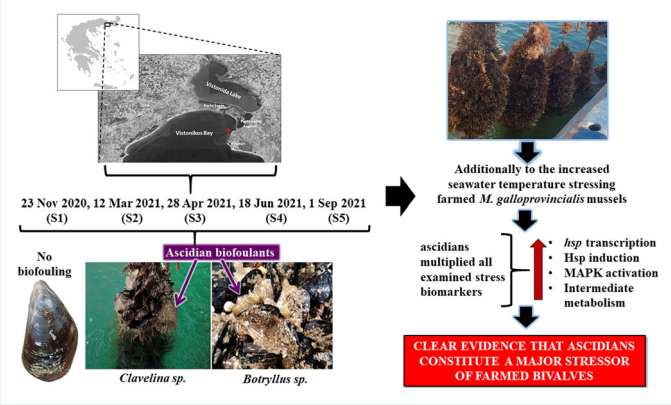
ABSTRACT

In biofouling communities, ascidians are among the most damaging species, presenting severe threats, such as depressed growth rates and decreased chances of lower survival, to shellfish aquaculture. However, little is known concerning the fouled shellfish physiology. In an effort to obtain information for the magnitude of stress caused by ascidians to farmed *Mytilus galloprovincialis,* five seasonal samplings took place in a mussel aquaculture farm suffering from ascidian biofoulants, in Vistonicos Bay, Greece. The dominant ascidian species were recorded and several stress biomarkers, including Hsp gene expression at both mRNA and protein levels, as well as MAPKs levels, and enzymatic activities of intermediate metabolism were examined. Almost all investigated biomarkers revealed elevated stress levels in fouled mussels compared to non-fouled. This enhanced physiological stress seems to be season-independent and can be attributed to the oxidative stress and/or feed deprivation caused by ascidian biofouling, thus illuminating the biological impact of this phenomenon.

GRAPHICAL ABSTRACT



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Biofouling; stress response; mussel farming; aquaculture; Ascidians

Introduction

There is a global need to sustainably expand aquaculture production in order to meet the demands of a growing human population (Willer and Aldridge 2019). Mollusk aquaculture, mainly consisting of bivalves (oysters, clams, scallops, mussels), is estimated to provide approximately 60% of the total marine aquaculture products worldwide (Yang 2021). However, the biofouling phenomenon is considered to be one major setback to rearing marine species (Dobretsov and Rittschof 2020; Liu et al. 2022). Biofouling is defined as the accumulation of various undesirable organisms in submerged surfaces. Biofouling communities may represent a considerable proportion of the total weight of typical aquaculture facilities (Woods et al. 2012). Shellfish aquaculture seems to be particularly vulnerable to biofouling due

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to the great number of artificial substrates; combined with the bivalve shells, these factos create extended underwater surfaces (McKindsey et al. 2007). In particular, suspended cultures, which can be free of predators, provide a safe area for fouling organisms to grow (Rocha et al. 2009). Moreover, it should be highlighted that many biofouling species constitute spatial competitors that can reach huge densities or biomass over short periods of time (Dealteris et al. 2004; Blum et al. 2007).

Biofouling can be a remarkable threat to marine aquaculture industry, since direct economic losses due to this phenomenon (Cahill et al. 2022) were estimated within the European aquaculture industry at 5–10% of the industry value (Lane and Willemsen 2004). Considering the final market price, biofouling was earlier calculated to cause economic losses of up to 20% for oyster (Enright 1993) and 30% for scallop (Claereboudt et al. 1994) aquaculture. In the U.S., bivalve growers claimed that approximately 15% of the total bivalve culture production cost was spent on biofouling management (Adams et al. 2011). However, biofouling cost often differs significantly between aquaculture locations and species, and is highly dependent on practices used, as farmers use different management techniques (Bannister et al. 2019).

Solitary ascidians grow as independent individuals and often form dense aggregations because of their high fecundity (Carver et al. 2003). Moreover, ascidians, due to their colonial nature, grow as sheet-like colonies of zooids immersed in a communal extracellular tunic. Several ascidians also exhibit strong invasive tendencies and behaviors, resulting in a growing awareness of the effects of invasive ascidians to benthic communities (Whitlatch et al. 1995; Lambert 2001). Life history traits of many ascidian species can explain their ability for invasion and successful colonization of a new territory. The capability of colonial species for regeneration from fragments, rapid growth, quick individual sexual maturity, hermaphroditic reproduction, long reproductive lifespan, huge fecundity and a dispersal larval phase are all favorable in their adaptability and rapid spread into new environments (Grosberg 1981; Kremer et al. 2010).

Studies have shown that ascidians downgrade shellfish fitness, and may also potentially smother or impede proper valve function (Jackson 1983; Lodeiros and Himmelman 1996; Woods et al. 2012; Ordóñez et al. 2016). For instance, *Didemnum vexillum* (Kott, 2002) is a colonial ascidian whose overgrowth in mussel ropes smothers mussels (Coutts and Forrest 2007). Many other colonial ascidians form large physical barriers that pevent shellfish valves from opening. This can result in reduced food availability (Riisgård et al. 1995) and possible reduced oxygen availability. Solitary ascidians are common colonists of mussel aquaculture worldwide. Specifically, *Ciona intestinalis* (Linnaeus, 1767) is a common problem for mussel aquaculture in the U.S. (Lesser et al. 1992), New Zealand (Woods et al. 2012) and Canada (Howes et al. 2007). The presence of this ascidian has been linked with increased mussel mortalities and depressed growth and overall fitness (Daigle and Herbinger 2009). *Styela clava* (Herdman, 1881) is also a familiar fouling organism in bivalve aquaculture (Lutz-Collins et al. 2009), severely harming mussel and oyster productivity since it can cover cultivation equipment (LeBlanc et al. 2007; Davis and Davis 2010). Therefore, it can be concluded that in shellfish aquaculture, ascidians may cause reduced growth and weight, poor condition indices, mortalities and worse flesh quality of the cultured species. Negative impacts on mussel farming are the result of interaction and strong competition between fouling species and mussels for limiting resources, such as food and space (Woods et al. 2012). Numerous studies have investigated the impacts of ascidian biofouling to cultured bivalve species. However, the majority of these studies (under both field and laboratory conditions) focus primarily on growth, condition indices and mortality of farmed species. Nevertheless, almost no attention has been paid to the effect of biofouling on the cellular physiology of fouled shellfish. The study of changes in the abundance of proteins and their post-translational modifications (early-warning indicators) has become a powerful tool for generating hypotheses regarding the manner in which environmental changes affect the biology of marine organisms, and can provide measurement endpoints that can be used in environmental management and ecological risk assessment (ERA) processes (Adams 1990). Bivalves, however, are additionally exposed to acute temperature raise of surface seawater, a result of the driving impacts of climate change in aquatic ecosystems, and therefore exhibit increased oxidative and anaerobiosis (Abele et al. 2002; Sokolova et al. 2012; Feidantsis et al. 2020). Specifically, several marine areas of the Mediterranean Sea have been identified as climate change "hotspots" with respective effects on marine biodiversity and productivity (Bethoux et al. 1990; Nicholls and Hoozemans 1996; Gambaiani et al. 2009; Calvo and Marsh 2011). In this context, and due to the lack of knowledge regarding the seasonal impact of ascidian biofouling on *Mytilus galloprovincialis*

(Lamarck, 1819), in this study, five seasonal samplings took place, and several biochemical and cellular indicators were examined in cultured Mediterranean mussels in order to investigate the hypotheses that: (1) ascidian biofouling impacts mussels' physiology; and (2) ascidian biofouling presents an extensive seasonality, further influencing this impact. For this reason, heat shock proteins (Hsp) gene expression and induction, along with key intermediate metabolism variations, were evaluated. Particularly, apart from Hsp, the phosphorylation of three important mitogen activated protein kinases' (MAPKs) family members (p38 MAPK, p44/42 MAPK and pJNKs) was assessed for two reasons: firstly, the MAPK pathway is especially activated by various stressful stimuli such as hypoxia (Gaitanaki et al. 2004; Anestis et al. 2010), thermal stress (Anestis et al. 2007; Anestis et al. 2008; Gourgou et al. 2010; Yao and Somero 2012), various pollutants (Châtel et al. 2010) and bacterial challenges (Zhang et al. 2018; Yang et al. 2020); and secondly, a clear connection between p38 MAPK phosphorylation and hsp70 and other cytoprotective genes has been previously revealed in *M. galloprovincialis* (Anestis et al. 2007; Gourgou et al. 2010).

Materials and methods

Sampling location and measurements of sea water temperature in the field

Five seasonal samplings were conducted during 2020–2021 in a *M. galloprovincialis* aquaculture farm in Vistonikos Bay (40°58′40.87″N, 25° 7′37.47″E), North Aegean, Greece. The culture site has an average depth of 8 m. The surrounding area includes three basins rich in organic material, namely Porto Lagos Lagoon, Vistonicos Gulf and Vistonida Lake (Figure 1) offering excellent conditions for bivalve farming (Georgoulis et al. 2022). Sampling dates were: 23 Nov 2020 (S1), 12 Mar 2021 (S2), 28 Apr 2021 (S3), 18 Jun 2021 (S4), 1 Sep 2021 (S5). Measurements of surface sea water temperature were performed using a Multiparameter Water Quality Meter (Model WQC-24, DKK-TOA Company). In addition to water temperature, salinity, dissolved oxygen concentration and pH were also recorded.

Sampling procedure

Samplings were conducted in a mussel aquaculture farm in which the longline culture system is used. Before the mussel collection, ascidian fauna was recorded. Ascidian presence's density was evaluated by visual observation of mussel culture sleeves. Specifically, density was divided in four categories, where '–' indicates the complete absence of ascidians, '− (→ +)' indicates the sporadic presence of ascidians on the mussel sleeve surface, '+' indicates the presence of ascidians covering less than 30% of the mussel sleeve surface, '++' indicates ascidians covering approximately 30–70% of the mussel sleeve surface, and '+++' indicates the presence of ascidians covering more than 70% of the surface. Four mussel sleeves were randomly selected and brought out of water, and ascidian species were recorded. Afterwards, fouled and non-fouled mussels were collected. Mussels covered from ascidian *Clavelina oblonga* (Herdman, 1880), as well as surrounded by 5 solitary ascidians [*Styela plicata* (Lesuer, 1823) at S2 and *S. plicata* along with *Ciona robusta* (Hoshino and Tokioka, 1967) at S1 and S5] inside a range of 20 cm around them, were considered fouled. Individuals without *C. oblonga* above them and without *S. plicata, C. robusta,* or any other ascidian 20 cm on all sides around them were marked as non-fouled (control) specimens. In samplings S3 and S4, *C. oblonga* was absent. Furthermore, richness and abundance of ascidians were very low; thus, only control mussels were collected. Only adult mussels (over 4.5 cm shell length) were sampled. Moreover, both fouled and non-fouled mussels were also free of other than ascidian fouling organisms (naked eye observation). From the five samplings conducted, 12 fouled and 12 control mussels were collected, 3 from each mussel sleeve. Right after collection, mussels were dissected, the mantle and posterior adductor muscle (PAM) tissues were isolated from the 24 individuals, and then flash frozen in liquid nitrogen until they were stored in the deep freezer (−80 °C).

Experimental analysis

RNA extraction and cDNA synthesis

Total RNA from mussel's mantle and PAM was extracted with the use of NucleoZOL reagent (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. The optional phase separation step was not performed and rest of actions followed the protocol. Approximately 50 mg of mantle and PAM from four specimens (one from each mussel sleeve) were pestle-homogenized in 500 µl NucleoZOL and RNAase-free water was added to the lysate. Subsequently samples were centrifuged and isopropanol was added to the supernatant to precipitate the RNA. Again, samples were centrifuged and two ethanol washes of the RNA pellet followed. Then, the pellet was diluted in 60 µl nuclease-free water. Extracted RNA stored at −80 °C, until reverse

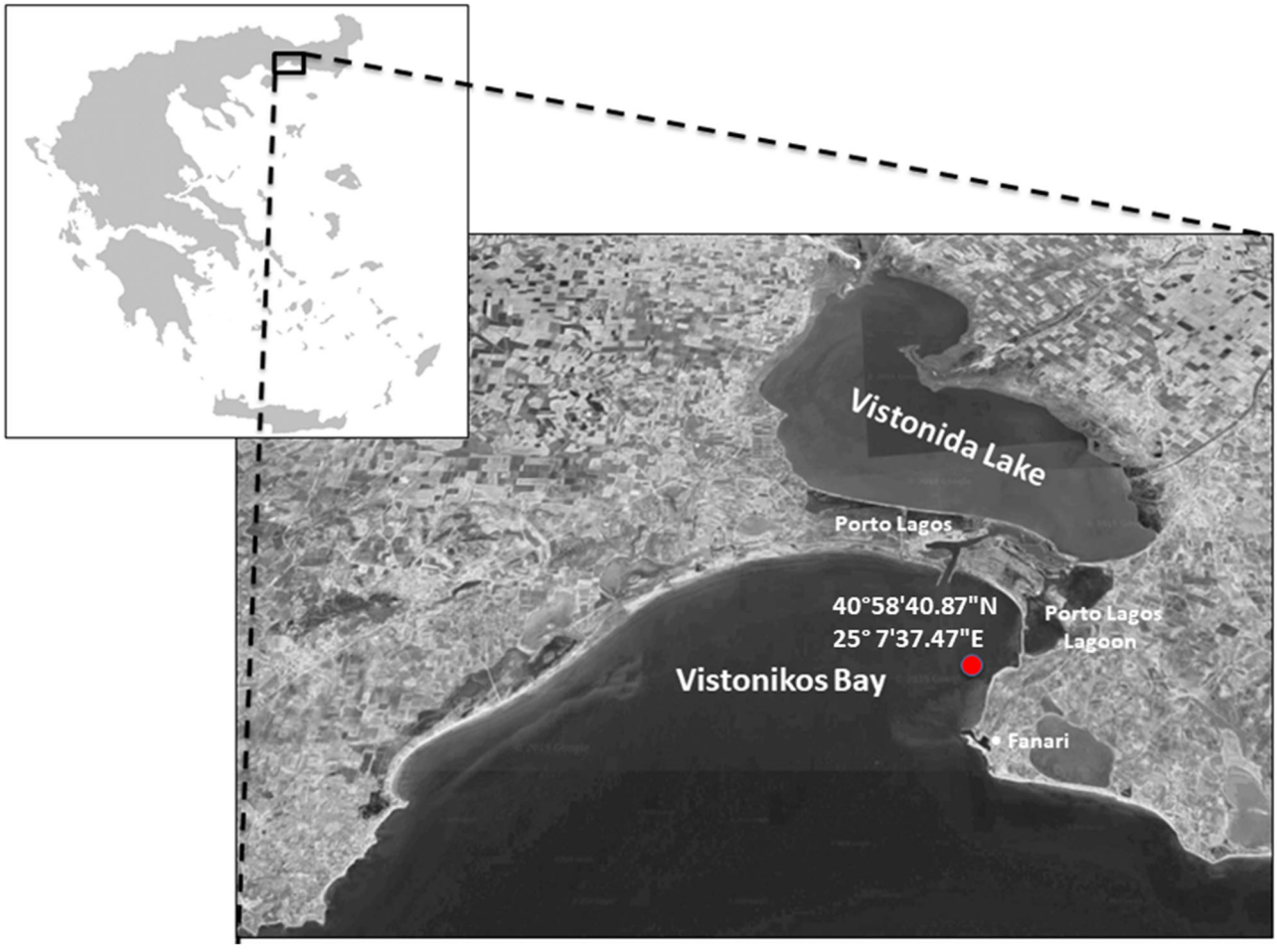

Figure 1. Sampling site in Vistonikos bay indicated by the red dot (40°58′40.87″N, 25° 7′37.47″E).

transcription. Before cDNA synthesis RNA concentration and purity were checked on a Quawell UV-Vis 5000 spectrophotometer (Quawell Technology, San Jose, CA, USA). For reverse transcription, about 500 ng of total RNA of each sample used in the reaction with PrimeScript kit (Takara, Japan) and the oligodT primers, according to the manufacturer's protocol. As with RNA, cDNA concentration and purity were measured and the samples were kept at −20 °C until qPCR application.

Gene expression analysis

Heat shock protein gene expression (*hsp70* and *hsp90*) was evaluated through quantitative real time PCR. The comparative CT method ($2^{-\Delta\Delta CT}$ method) as described by Livak and Schmittgen (2001) was applied to quantify the relative expression of the two genes in mantles and PAMs of mussels. Mantle and PAM cDNA of non-fouled animals were the control samples and target gene expressions were normalized to the most stable reference gene (beta actin for posterior adductor muscle and elongation factor 1 alpha for mantle). For each gene, four mussel cDNA from every sampling were run in qPCR. PCR reactions performed with KAPA SYBR® FAST qPCR Master Mix (2×) kit, in 10 µl final volume. Each sample contained 10 ng of mussel cDNA as template, 5 µl of KAPA SYBR® FAST qPCR Master Mix (2×), 2 µM of the two primers and PCR-grade water up to 10 µl. Runs were carried out in qPCR Thermocycler Eco 48 Real-time PCR (Illumina) for 40 cycles. Primers used are listed in Table 1.

SDS-PAGE/immunoblot

Mantle and PAM tissue preparation was performed as follows. Approximately 100 mg of tissue were homogenized in 1/3 *w*/*v* lysis buffer (20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0.2 mM Na_3VO_4, 10 mM benzamidine, pH 7.0, containing 200 µM leupeptin, 10 µM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 5 mM dithiotheitol, 300 µM PMSF, 50 µg mL^{-1} pepstatin and 1% *v*/*v* Triton X-100). Following a 30 min extraction on ice, samples were centrifuged at 10,000*g* for 10 min at 4 °C and then supernatants were boiled with 0.33 volumes of sample buffer (330 mM

Table 1. List of primers used in this study, amplicon size and Genbank accession number of target sequence.

Target gene	Forward primer (5′-3′) Reverse primer (5′-3′)	Amplicon (bp)	GenBank accession no.	Reference
hsp70	5′-CGGAGGCAAGCCAAAACTAC-3′ 5′-AGCCTCGGCAGTTTCTTTCA-3′	109	AB180909.1	Giannetto et al. (2017)
hsp90	5′-TGCTGATAAAGTAGTTGTCCA-3′ 5′-CTGACTGTGTTTCTTCACAAC-3′	208	AJ586906	This study
β-actin	5′-CGACTCTGGAGATGGTGTCA-3′ 5′-GCGGTGGTTGTGAATGAGTA-3′	153	AF157491.1	Moreira et al. (2014)
EF-1α	5′-GATATGCGCCAGTCTTGGAT-3′ 5′-CTCATGTCTCGGACAGCAAA-3′	223	AB162021	Moreira et al. (2014)

Tris-HCl, 13% *v*/*v* glycerol, 133 mM DTT, 10% *w*/*v* SDS, 0.2% *w*/*v* bromophenol blue). Protein concentrations were measured using the BioRad protein assay. Then, equal amounts of proteins (80 µg) were separated on 10% (*w*/*v*) acrylamide and 0.275% (*w*/*v*) bisacrylamide slab gels, and transferred electrophoretically onto nitrocellulose membranes (0.45 µm, Schleicher & Schuell, Keene N. H. 03431, USA). Non-specific binding sites on the membranes were blocked with 5% (*w*/*v*) non-fat milk in TBST (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (*v*/*v*) Tween 20) for 30 min at room temperature. Afterwards, nitrocellulose membranes were subjected to overnight incubation with the appropriate primary anti-bodies: mouse anti-hsp70 (H5147, Sigma, Germany), monoclonal mouse anti-hsp90 (H1775, Sigma, Germany), monoclonal rabbit anti-phospho p44/42MAPK (Thr202/Tyr204) (4376, Cell Signaling, Beverly, MA, USA), polyclonal rabbit anti-phospho-p38 MAP kinase (Thr180-Tyr182) (9211, Cell Signaling, Beverly, MA, USA) and monoclonal mouse anti-phospho-SAPK-JNK (Thr183-Tyr185) (Cell Signaling, Beverly, MA, USA). Quality transfer and protein loading were assured by Ponceau stain. After washing in TBST (3 times, 5 min each), the blots were incubated with horseradish peroxidase-linked secondary anti-bodies, washed once more 3 times for 5 min in TBST and the bands were detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, GraphPad, San Diego, CA, USA).

Metabolic enzyme activities in the tissue homogenates

Enzyme activity assay performed for metabolic enzymes L-lactate dehydrogenase (L-LDH, EC 1.1.1.27), Citrate Synthase (CS, EC 4.1.3.7) and 3-hydroxyacyl-CoA dehydrogenase (HOAD, EC1.1.1.35). Mantle and PAM homogenization for enzyme activity assessment were prepared as described by Driedzic and Almeida-Val (1996) and Speers-Roesch et al. (2016). Regarding L-LDH, and HOAD the tissues were homogenized in a buffer containing 150 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} dithiothreitol (DTT) and 1% Triton X-100, pH 7.4. For CS, tissues were homogenized in a buffer containing 20 mmol l^{-1} HEPES, 1 mmol l^{-1} EDTA with 1% Triton X-100, pH 7.4. Following homogenization samples were centrifuged at 13,000*g* for 10 min in 4 °C and supernatants used for enzymatic assays.

L-LDH activity was determined in a medium containing 0.15 mmol l^{-1} NADH, 1 mmol l^{-1} KCN and 50 mmol l^{-1} imidazole, pH 7.4. The reaction was triggered by the addition of 1 mmol l^{-1} pyruvate (omitted for control). HOAD activity was determined in a medium containing 0.15 mmol l^{-1} NADH, 1 mmol l^{-1} KCN, 1 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.4. The reaction was triggered by the addition of 2.0 mmol l^{-1} acetoacetyl CoA (omitted for control). CS activity was determined in a medium containing 0.4 mmol l^{-1} acetyl CoA, 0.25 mmol l^{-1} DTNB and 75 mmol l^{-1} tris buffer, pH 8.0. The reaction was initiated by adding 0.5 mmol l^{-1} oxaloacetate (omitted for control).

Enzymatic activities were determined at 18 °C spectrophotometrically and all assessments are based on well-established protocols (Speers-Roesch et al. 2016). L-LDH, and HOAD activities were measured following the oxidation of NADH at 340 nm (extinction coefficient, $\varepsilon = 6.22\ mM^{-1}\,cm^{-1}$) and CS enzyme activity were determined using the reaction of free coenzyme A with 5.5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm ($\varepsilon = 13.6\ mM^{-1}cm^{-1}$). All enzyme activities expressed as µmoles of substrate min^{-1} g^{-1} wet tissue.

Statistical analysis. Normality of data was tested using Ryan-Joiner's test, followed by Levene's test for testing the homogeneity of variances of the means. Afterwards, one-way analysis of variance (ANOVA) (GraphPad Instat 3.0) performed at $p < 0.05$ (5%) level between all experimental groups examined herein (S1: fouled, S1: non-fouled, S2: fouled, S2: non-fouled, S3: non-fouled, S4: non-fouled, S5: fouled, S5: non-fouled). Finally, post hoc Tukey's HSD test was performed to define the statistically significant differences ($p < 0.05$) between the means of the

different groups. The effects of biofouling and its seasonal impact were statistically compared, applying a Nested model ANOVA (SPSS Scientific Inc. Software, version 21). Values are presented as means ± SD.

Results

Ascidian fauna in the study area

Table 2 shows the recorded major ascidian fauna species observed in the study area in the different sampling dates. Due to the extremely low density of other ascidian species on mussel sleeves, they were considered as non-significant biofoulers, and therefore were not included. Moreover, the intensity of ascidian fouling in mussels exceeded 70% of the mussel sleeve surface in S1, S2 and S5, while on the other hand, in samplings S4 and S5, the presence of ascidians was either non or extremely sporadic, and under this prism it was characterized as − (→ +) (Table 2).

Clavelina oblonga exhibited the greatest densities in samplings S1 (Figure 2A) and S5, low densities in sampling S2, and was absent from samplings S3 and S4. *Styela plicata* was present in all samplings in the study area with moderate (S3, S4) to high densities (S1, S2, S5). *C. robusta* exhibited moderate densities only in S1 and S5, whereas *Botryllus* sp. (Figure 2B) exhibited moderate abundance in samplings S2 and S3. Salinity, dissolved oxygen concentration, and pH presented minimal fluctuations in the five samplings. Mean sea surface temperature was as follows: S1 (23 Nov 2020): 18.4 °C, S2 (12 Mar 2021): 12.5 °C, S3 (28 Apr 2021): 15.3 °C, S4 (18 Jun 2021): 23.8 °C and S5 (1 Sep 2021): 27.2 °C.

hsp70 and *hsp90* mRNA expression

In general, fouled mussels exhibited significantly ($p < 0.05$) greater *hsp70* and *hsp90* mRNA expression compared to non-fouled individuals. While non-fouled mussels exhibited the highest levels of mRNA expression in S4 when the highest ambient seawater temperature was recorded, fouled mussels exhibited the opposite pattern, with the highest levels exhibited in S1. Regarding seasonality, the difference in this parameter between mussels with and without ascidian biofouling was at a maximum in S1, less in S5 and lesser in S2. Moreover, in S1, *hsp70* and *hsp90* mRNA expression was significantly ($p < 0.05$) elevated in the mantle of fouled mussels when compared with non-fouled individuals. In the mantle, as far as *hsp90* mRNA expression is concerned, the difference in seasonality between mussels with and without ascidians biofouling was at a maximum in S1 and less in S2 (Figure 3A,C). Concerning PAM, *hsp70* mRNA expression was also statistically significantly ($p < 0.05$) increased in fouled mussels compared to the non-fouled ones. As far as seasonality is concerned, the difference in this parameter between mussels with and without ascidian biofouling was at a maximum in S1, less in S2 and lesser in S5. However, *hsp90* mRNA expression was similar in both fouled and non-fouled individuals (Figure 3B,D). In S2, *hsp90* mRNA expression exhibited similar expression patterns with no statistically significant differences between fouled and non-fouled mussels in both examined tissues. Moreover, *hsp70* mRNA expression was higher in the mantle compared to the PAM. In S5, significantly higher ($p < 0.05$) expression levels of *hsp* genes were detected in both tissues of fouled mussels compared to the non-fouled individuals (Figure 3C,D).

Hsp70 and Hsp90 induction

In contrast to *hsp* mRNA expression, Hsp induction exhibited a more apparent pattern among fouled and non-fouled (control) mussels in all sampling dates. The highest Hsp70 and Hsp90 levels were observed in non-fouled mussels with increasing and increased ambient sea water temperature. A similar pattern was observed in fouled mussels, with the highest levels observed in S5. Both in the mantle and PAM, the induction levels of both Hsps were significantly

Table 2. Major ascidian species detected in the study and ascidian presence density in the different sampling dates.

Ascidian species[a]	23 Nov 2020 (S1)	12 Mar 2021 (S2)	28 Apr 2021 (S3)	18 Jun 2021 (S4)	1 Sep 2021 (S5)
Styela plicata	+	+	+	+	+
Clavelina oblonga	+	+	–	–	+
Ciona robusta	+	–	–	–	+
Botryllus sp.	–	+	+	–	–
Ascidian presence[b]	+++	+++	− (→ +)	− (→ +)	+++

[a](+) indicates presence and (–) absence of ascidian species.
[b](–) indicates the complete absence of ascidians, [− (→ +)] indicates the sporadic presence of ascidians on the mussel sleeve surface, (+) indicates the presence of ascidians covering less than 30% of the mussel sleeve surface, (++) indicates ascidians covering approximately 30–70% of the mussel sleeve surface and (+++) indicates the presence of ascidians covering more than 70% of the mussel sleeve surface.

Figure 2. *Clavelina* sp. remarkable densities met in the sampling site in S1 (A) and *Botryllus* sp. in S3 (B).

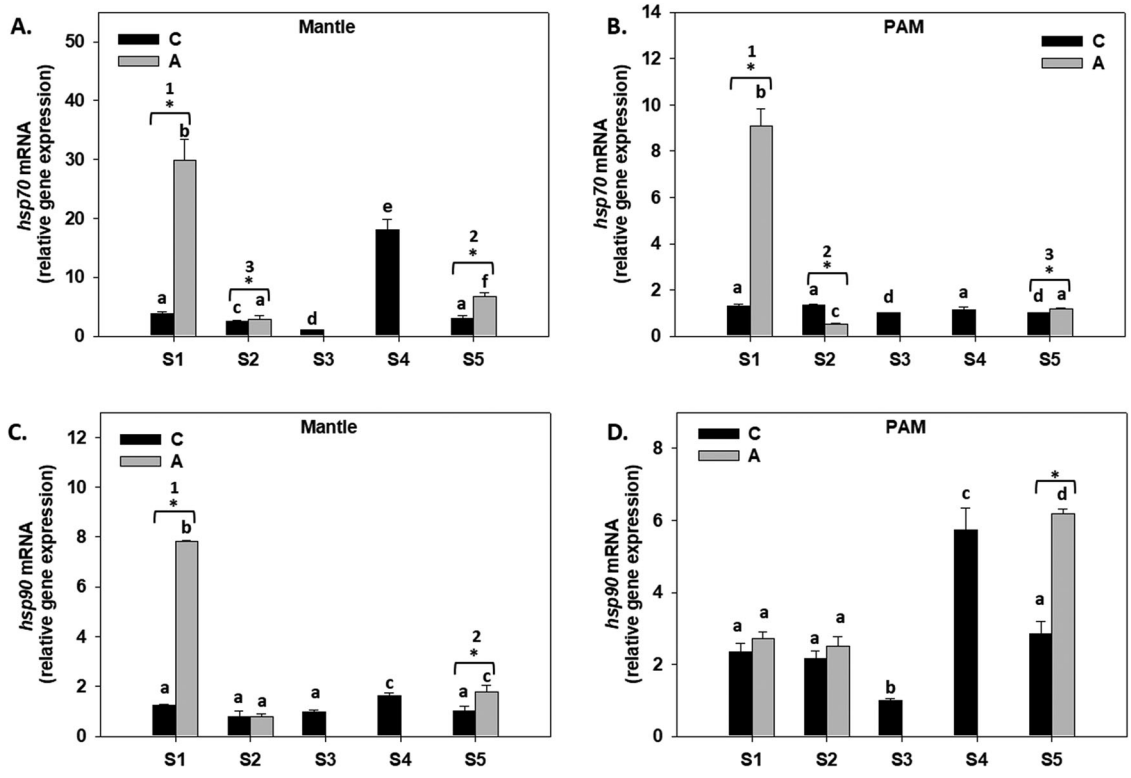

Figure 3. Relative mRNA levels of heat shock proteins genes *hsp70* (A, B) and *hsp90* (C, D) in mantle and posterior adductor muscle (PAM) of *Mytilus galloprovincialis* in the five samplings [23 Nov 2020 (S1), 12 Mar 2021 (S2), 28 Apr 2021 (S3), 18 Jun 2021 (S4), 1 Sep 2021 (S5)]. Vertical bars represent the mean ± standard deviation ($n = 4$) values from different animals. Lower case letters represent statistically significance differences at $p < 0.05$ between the different groups, asterisk (*) depicts statistically significant differences ($p < 0.05$) between fouled (A) and non-fouled mussels (C), while numbers depict seasonally significant differences of ascidian biofouling when comparing fouled (A) to the respective non-fouled mussels (C) ($1 > 2 > 3$).

($p < 0.05$) elevated in fouled mussels (Figure 4). Specifically, in S1, Hsps levels exhibited a similar pattern to that of their gene expression, with both Hsp70 and Hsp90 levels being significantly higher ($p < 0.05$) in the fouled compared to the non-fouled mussels. Similarly, in S2, Hsps levels were significantly higher ($p < 0.05$) in fouled individuals. In accordance with their gene expression, Hsp70 and Hsp90 induction levels were both elevated ($p < 0.05$) in both examined tissues of fouled mussels of S5, compared to the non-fouled individuals. Regarding seasonality, as far as Hsp70 levels in the mantle and PAM are concerned, the difference in this parameter between mussels with and without ascidian biofouling was at a maximum in S2, and less in S1 and S5 (Figure 4A,B). Hsp90, however, exhibited a different pattern, with difference in

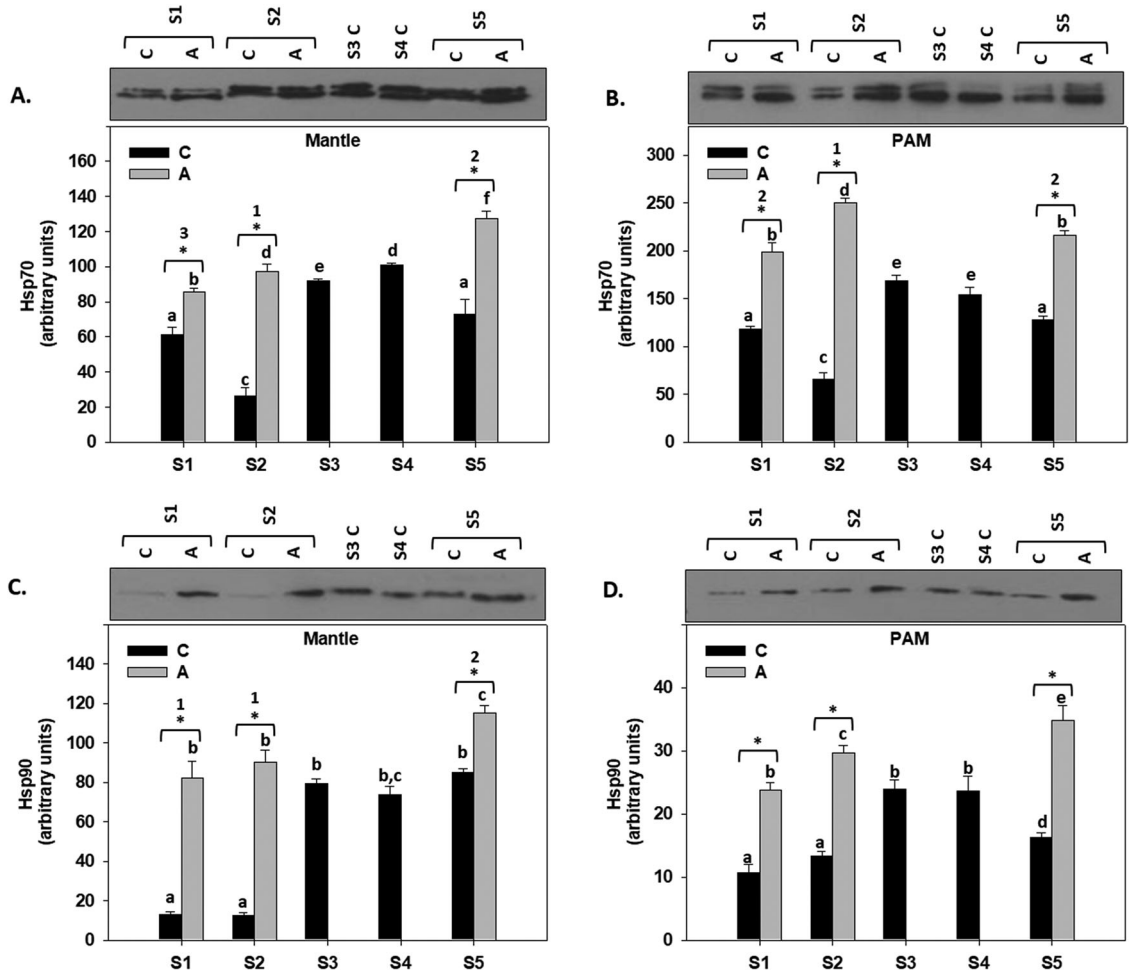

Figure 4. Hsp70 (A, B) and Hsp90 (C, D) levels in mantle and posterior adductor muscle (PAM) of *Mytilus galloprovincialis* in the five samplings [23 Nov 2020 (S1), 12 Mar 2021 (S2), 28 Apr 2021 (S3), 18 Jun 2021 (S4), 1 Sep 2021 (S5)]. Blots were quantified by laser-scanning densitometry and plotted. Vertical bars represent the mean ± standard deviation ($n = 4$) values from different animals. Lower case letters represent statistically significance differences at $p < 0.05$ between the different groups, asterisk (*) depicts statistically significant differences ($p < 0.05$) between fouled (A) and non-fouled mussels (C), while numbers depict seasonally significant differences of ascidian biofouling when comparing fouled (A) to the respective non-fouled mussels (C) ($1 > 2 > 3$).

seasonality between mussels with and without ascidian biofouling at a maximum in S5, less in S1 and lesser S2 in the mantle, while no seasonal differences were observed in the PAM (Figure 4C,D).

MAPK phosphorylation levels

As with Hsps, phosphorylated p38 MAPK, p44/42 MAPK and JNKs levels were statistically higher ($p < 0.05$) in fouled mussels compared to non-fouled ones in all samplings (Figure 5). While p44/42 MAPK in the mantle and PAM and JNKs in the mantle exhibited the highest phosphorylated levels in S3 and S4, during increasing and at increased water temperature, the opposite pattern was observed for p38 MAPK in the mantle and the PAM, and JNKs in the PAM. Considering the mantle of fouled specimens, all three kinases exhibited the highest phosphorylation levels in S5 (Figure 5A,C,E), while in the PAM, the highest phosphorylation levels in fouled individuals were observed in S2 and S5 (Figure 5B,D,F). Regarding seasonality, in both examined tissues the difference in p38 MAPK phosphorylation between mussels with and without ascidian biofouling was at a maximum in S2, less in S5 and lesser in S1 (Figure 5A,B). With reference to p44/42 MAPK phosphorylation, the difference in seasonality between mussels with and without ascidian biofouling was at a maximum in S5, less in S2 and lesser in S1, in the mantle, while in the PAM it was at a maximum in S2 and S5, and less in S1 (Figure 5C,D). Finally, regarding seasonality, JNKs exhibited increased phosphorylated levels in both examined tissues; the difference in this parameter between mussels with and without ascidian biofouling was observed at a maximum in S5, and less in S1 and S2 (Figure 5E,F).

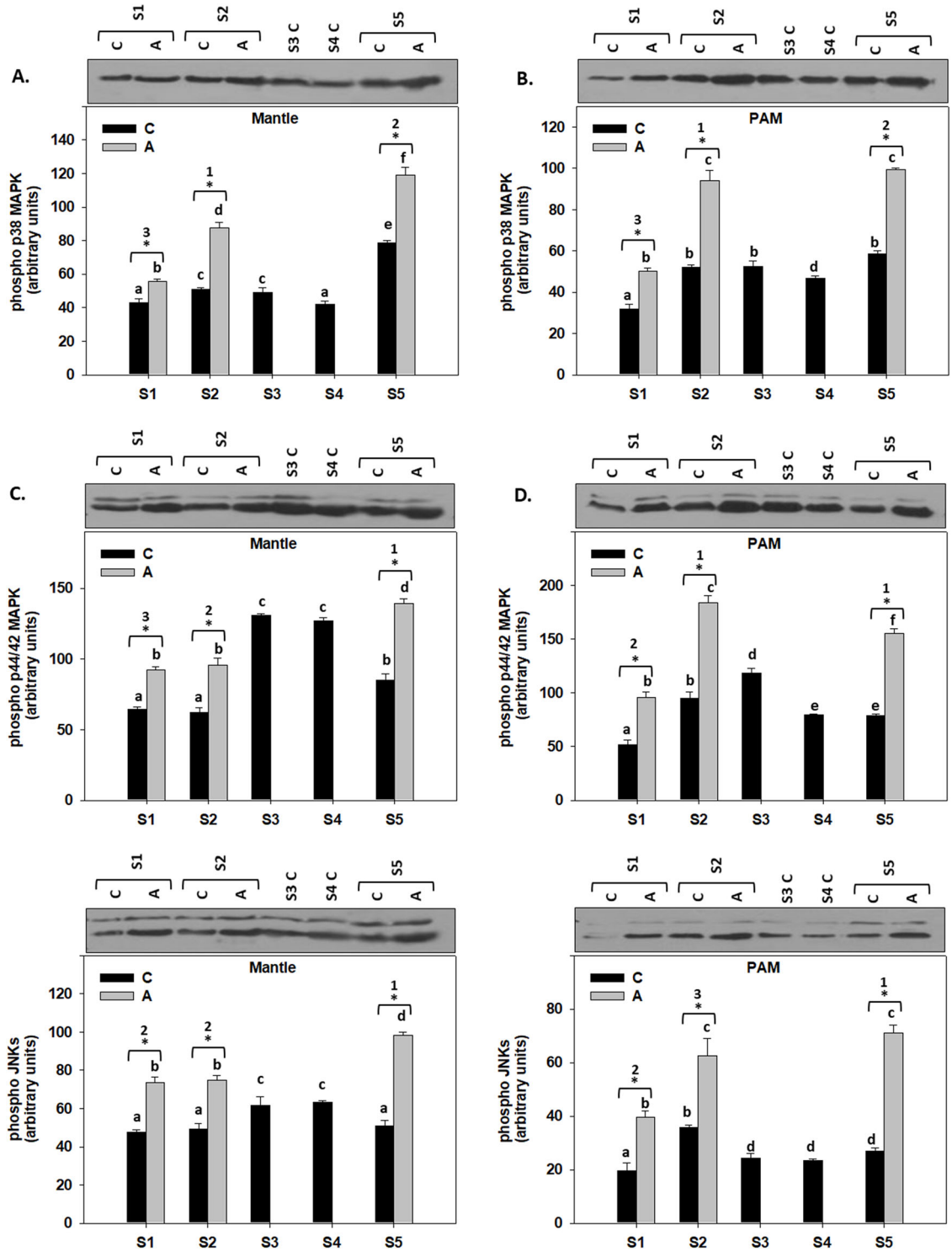

Figure 5. Phosphorylation levels of p38 MAPK (A, B), p44/22 MAPK (C, D) and JNKs (E, F) in mantle and posterior adductor muscle (PAM) of *Mytilus galloprovincialis* in the five samplings [23 Nov 2020 (S1), 12 Mar 2021 (S2), 28 Apr 2021 (S3), 18 Jun 2021 (S4), 1 Sep 2021 (S5)]. Blots were quantified by laser-scanning densitometry and plotted. Vertical bars represent the mean ± standard deviation ($n = 4$) values from different animals. Lower case letters represent statistically significance differences at $p < 0.05$ between the different groups, asterisk (*) depicts statistically significant differences ($p < 0.05$) between fouled (A) and non-fouled mussels (C), while numbers depict seasonally significant differences of ascidian biofouling when comparing fouled (A) to the respective non-fouled mussels (C) ($1 > 2 > 3$).

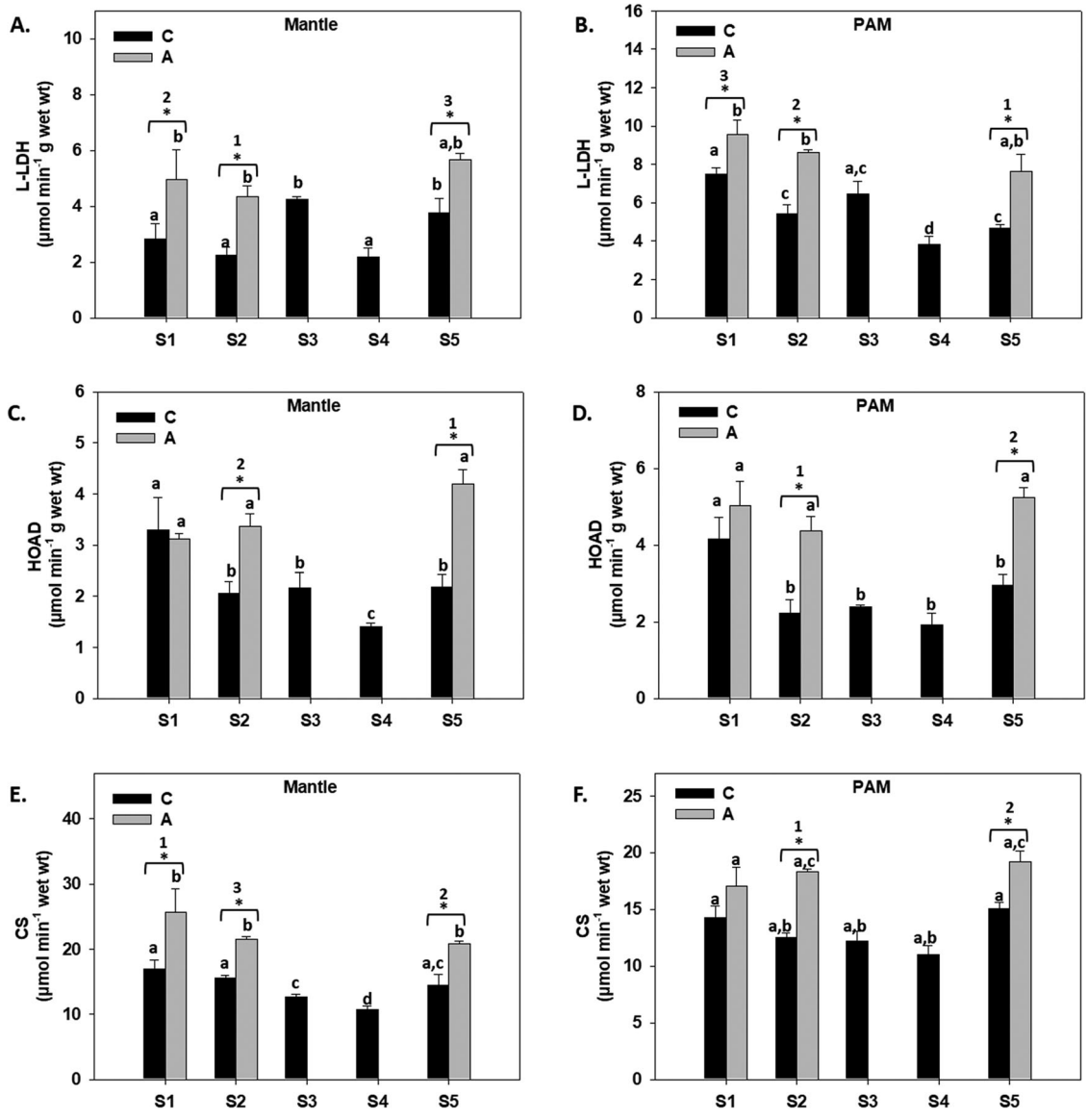

Figure 6. Changes in the activity of L-lactate dehydrogenase (L-LDH) (A, B), 3-hydroxyacyl-COA dehydrogenase (HOAD) (C, D) and citrate synthase (CS) (E, F) in mantle and posterior adductor muscle (PAM) of *Mytilus galloprovincialis* in the five samplings [23 Nov 2020 (S1), 12 Mar 2021 (S2), 28 Apr 2021 (S3), 18 Jun 2021 (S4), 1 Sep 2021 (S5)]. Vertical bars represent the mean ± standard deviation ($n = 4$) values from different animals. Lower case letters represent statistically significance differences at $p < 0.05$ between the different groups, asterisk (*) depicts statistically significant differences ($p < 0.05$) between fouled (A) and non-fouled mussels (C), while numbers depict seasonally significant differences of ascidian biofouling when comparing fouled (A) to the respective non-fouled mussels (C) (1 > 2 > 3).

Intermediate metabolism enzymatic activity

Examined metabolic enzymes generally displayed statistically significant higher ($p < 0.05$) activity levels in both tissues of fouled *M. galloprovincialis* regardless of the sampling time compared to non-fouled individuals (Figure 6). Exceptions include HOAD in the mantle and CS in the PAM in S1, when these enzymes presented statistically similar activity levels between fouled and non-fouled mussels. While no statistical significant differences were found in the fouled mussels between different samplings, in the non-fouled individuals the lowest enzymatic activity levels were exhibited mostly in S4, depicting an inversely proportional relation with increasing sea water temperature. As far as L-LDH is concerned in the mantle, and considering seasonality, the difference in this parameter between mussels with and without ascidian biofouling was at a maximum in S2, less in S1 and lesser in S5, while in the PAM the pattern S5 > S2 > S1 was observed (Figure 6A,B). With reference to HOAD activity levels, the difference in seasonality between mussels with and

without ascidian biofouling was at a maximum in S5 and less in S2 in the mantle, while in the PAM it was at a maximum in S2, and less in S5 (Figure 6C,D). While PAM exhibited the same seasonal pattern regarding CS activity levels compared to PAM HOAD, in the mantle, difference in seasonality between mussels with and without ascidian biofouling was at a maximum in S1, less in S5 and lesser in S2 (Figure 6E,F).

Nested ANOVA results

Table 3 demonstrates that the effect of both ascidian biofouling and season were statistically significant.

Discussion

This study reveals that biofouling ascidians may constitute a serious source of stress to cultured mussels *M. galloprovinciallis*, as indicated by all biological markers examined herein. Taking into consideration the fact that ascidians cause hypoxia to fouled mussels, and that hypoxic conditions favor the increase of reactive oxygen species (ROS) by multiple cellular systems, increased oxidative stress (Hitchon and El-Gabalawy 2004; Quiñonez-Flores et al. 2016) could be the factor triggering the Hsps induction in fouled mussels in every sampling, compared to control mussels. Hsps mainly prevent the formation of non-functional cellular proteins and assist protein folding under stressors such as temperature extremes, low oxygen levels, oxidative stress, pathogens and heavy metal exposure (Anestis et al. 2010; Liu et al. 2014; You et al. 2013; Liu et al. 2016; Feidantsis et al. 2020). However, the effect that ascidians pose to heat shock response (HSR) due to feed restriction should be further considered. Although no data exists in bivalve species, it has been shown in other marine organisms, such as teleosts, that starvation significantly increases hepatic Hsp70 and Hsp90 levels in *Labeo rohita* (Hamilton, 1822) fingerlings (Yengkokpam et al. 2008). Thus, it cannot be concluded whether the oxidative or the starvation stress caused by ascidian biofouling is the main trigger of HSR. Most likely, a synergistic effect of both the above is reflected in fouled mussels' response. Protein levels of both Hsps examined in this study were up regulated Moreover, apart from the post translational levels examined in this study, mRNA expression was also determined in order to assess the transcription stage of the protein expression process. In the case of mRNA expression, *hsp70* and *hsp90* exhibited some discrepancies to the pattern of Hsps levels. The discrepancy between transcriptional and post-translational levels in the proteins measured in this study can be attributed to the fact that mRNA levels do not accurately predict protein levels in eukaryotic cells. The latter is the case for this study, which exhibits a weak correlation between transcription and translation products' levels, and therefore, the results herein are in compliance with recent studies on eukaryotic cells which exhibit a discrepancy between mRNA and protein levels (Kolkman et al. 2006; Brockmann et al. 2007; Ingolia et al. 2009).

In addition to Hsps, MAPKs also possess a central role to mussels' responses when exposed to a great variety of stressors. Generally, after activation, MAPKs partially translocate to the cell nucleus, where they interact with several transcription factors, triggering the beginning of survival or apoptotic mechanisms (Bogoyevitch 2000). p38 MAPK has been linked to the mediation of cytoprotective genes (such as mt-20 and hsp70) (transcriptional induction along with delayed induction of apoptosis) after acute heat stress in *M. galloprovincialis* (Gourgou et al. 2010). All three MAPKs phosphorylated levels were found significantly up regulated in fouled mussels regardless of the sampling time, indicating a season-independent elevated stress in fouled specimens. Anestis et al. (2007) reported increased p38 MAPK phosphorylation in parallel with increased expression of *hsp* genes in mussels exposed to temperatures above 24 °C. The present results are in line with this pattern in warmer seawater (S5—27.2 °C) but also at temperatures of 12.5 °C (S2) and 18.4 °C (S1). These results indicate that elevated levels of all three MAPKs and Hsps among fouled individuals are not driven solely by temperature extremes, reinforcing the role of ascidians in the stress. Ascidian epibiotic pressure on the mussels studied herein, may be focused on the anaerobic conditions these organisms generate. Feidantsis et al. (2020) have shown that gene expression and an increase in the activities of antioxidant enzymes and

Table 3. Results of nested ANOVA analyses.

hsp genes	SS	df	MS	*F*	*p*
Season	256.72	2	113.44	38.199	0.035*
Biofouling (season)	315.28	4	156.70	54.301	0.029*
HSR					
Season	322.76	2	156.96	47.617	0.019*
Biofouling (season)	435.08	4	187.66	51.202	0.011*
MAPK					
Season	299.81	2	122.98	43.003	0.022*
Biofouling (season)	447.88	4	188.98	58.873	0.009*
Intermediate metabolism					
Season	311.98	2	132.44	43.987	0.025*
Biofouling (season)	443.44	4	180.99	54.664	0.010*

*Indicates statistically significant effect

Hsps after thermal exposure of *M. galloprovincialis* might trigger ROS production, which is closely correlated with anaerobic metabolism under hypometabolic conditions. Similarly, the MAPKs' increased phosphorylation studied herein could be attributed to the hypoxic conditions and the subsequent oxidative stress fouled mussels are subjected to. It is well-documented that oxidative stress plays a key role in MAPK activation (Takata et al. 2020). Similar to the HSR exhibited herein, the phosphorylation of MAPKs could be also attributed to the feed restriction that fouled mussels are exposed to due to biofouling. Antonopoulou et al. (2013) have shown that starvation affects MAPK activation by generally increasing it in several tissues of the European Sea Bass (*Dicentrarchus labrax*) (Linnaeus, 1758). It can be assumed that the activation of both HSR and MAPK act cyto-protectively in order to avoid the induction of cell death pathways, and therefore, sustain a certain level of homeostasis in the fouled mussels. Although both these processes are energetically expensive and fouled individuals are deprived of nutrients, it seems that fuel stores are deviated to cytoprotection. However, the latter is just a hypothesis and requires further investigation.

Despite the fact that ascidians are epibiotic organisms, their effect on mussels could be compared to parasitic relationships. In such relationships s, Hsp60 and Hsp70 levels were increased from the cell fraction of peripheral blood obtained from nestling house martins (*Delichon urbica*) (Linnaeus, 1758) and adult barn swallows (*Hirundo rustica*) (Linnaeus, 1758) in parasite-infested nests, exhibiting a key role of Hsps in host-parasite interactions (Merino et al. 1998; Merino et al. 2002). Moreover, in the carob moth larvae *Ectomyelois ceratoniae* (Zeller, 1839), parasitism by wasps caused increased *hsp70* and *hsp90* gene expression (Farahani et al. 2020). Farahani et al. (2020) suggest that the oxidative stress induced by parasitism and its subsequent damage are reduced by Hsp mediation in *E. ceratoniae*. Additionally, MAPKs are also involved in host-parasite interactions in both plant and animal organisms. Specifically, *Giardia intestinalis*-induced inflammatory response levels in mouse macrophages are mediated through activation of the p38 MAPK, p44/42 MAPK and NF-κB signaling pathways (Zhao et al. 2021).

While several investigations revealed up-regulation of energy metabolism genes in mussels during thermal stress (Gracey et al. 2008), the results herein indicate that epibiotic pressure due to ascidian fauna triggers metabolic processes in order for fouled mussels to exploit all available energy deposits. Specifically, fouled mussels presented similar activities to all seasonal samplings, presenting a season-independent enhanced glycolitic rate, impaired aerobic capacity (L-LDH), and enhanced oxidation of fatty acids that might contribute to ATP turnover (HOAD) compared to non-fouled mussels. The increases in the activities of glycolytic enzymes and PK during exposure of *M. galloprovincialis* to temperatures higher than 26 °C clearly support glycolysis reactivation (Anestis et al. 2007; Ioannou et al. 2009; Feidantsis et al. 2020). Specifically, Anestis et al. (2007) have found that this exposure to thermal stress leads these organisms to valve closure, and therefore mussels become anaerobic and possibly feed-deprived. The latter mimics the condition to which fouled mussels are exposed in this study. Moreover, this metabolic response is closely correlated to the increased *hsp70* and *hsp90* gene expression levels after thermal exposure, suggesting that the energy-demanding process of Hsps synthesis is met by reactivation of glycolytic ATP production in the tissues of both laboratory-acclimated and naturally acclimatized *M. galloprovincialis* at temperatures higher than 26 °C, which supplements previous observations (Feidantsis et al. 2020). Although in the current study CS activity was similar in varying water temperatures (12.5–27.2 °C), its increased aerobic capacity (CS) in the fouled mussels indicates that aerobic metabolism is also activated, although oxygen availability is minimized in these individuals. However, marine species can be very vulnerable to alterations in oxygen availability (Sokolova and Pörtner 2003). CS synthase is a key enzyme of aerobic ATP-producing pathways and usually used as an indicator of aerobic capacity in invertebrates. This index commonly increases at higher temperatures in many ectotherm species (Crockett and Sidell 1990; Sokolova and Pörtner 2003; Lesser and Kruse 2004), but several species of marine molluscs do not follow this pattern (Bjelde and Todgham 2013; Collins et al. 2020; Torossian et al. 2020). Thus, increased metabolic activities of fouled mussels in this study may indicate increased overall maintenance energy costs and ATP generation, regardless of the temperature. Considering the possible stressors, with the exception of the abiotic factors, many biological interactions may also stress an organism (fouling, disease, space and food competition and predation). Interactions between species can become more complicated, after the introduction of non-indigenous species like ascidians, and may lead to a severe disruption of well-established networks (Strayer et al.

2006). Ascidians, as active filter feeders, process and filter large volumes of water. *Styela plicata*, a very abundant ascidian in all seasons in the study area in Vistonikos Bay, was found in laboratory aquaria to possess a filtration rate of over 6 l h^{-1} (Fiala-Médioni 1978). LeBlanc et al. (2007) reported a remarkable decline in stock weights due to *S. clava* presence. Although *C. intestinalis* (*C. robusta* detected at Vistonikos Bay) did not directly compete for food with *Mytilus edulis* (Linnaeus, 1758) in a mussel aquaculture farm (Lesser et al. 1992), this species represents an imposing problem for mussel farmers on the Atlantic coast of Canada (Howes et al. 2007). Mussels fouled with the ascidians *C. intestinalis*, *Ectopleura crocea* (Agassiz, 1862), *S. clava* and *D. vexillum* exhibited significantly reduced condition indexes and also depressed growth of shell and flesh (Auker 2010; Sievers et al. 2013). Concerning *C. oblonga*, extreme densities were found in the study area, which may suffocate several mussels due to great physical barrier formation. Similarly, Ordóñez et al. (2016) recorded smothered mussel spat by *C. oblonga* along the Mediterranean coast of Spain. Under stress, organisms trigger physiological trade-offs, whereby energy is allocated aside from growth and reproduction, as the organism focuses on physiological defenses that improve their immediate odds of survival. Thus, increased stress due to fouling as revealed by the biochemical markers examined in this study could possibly be accompanied by the poor performance found in the aforementioned studies.

Apart from the complications caused to the cultured species, ascidians colonize the equipment used for mussel cultivation and become injurious to farm's productivity (LeBlanc et al. 2007). The aquaculture industry often uses extreme approaches to mitigate biofouling, many of which also harm the cultured organism. *In situ* treatment methods, in coordination with effective, cheap and easy removal, are essential, ideally in combination with minimal environmental impact. In Greece, many mussel farmers expose the mussels for one or two days to air. According to the current observations, it seems that there is a great reduction in ascidian fauna following this technique. LeBlanc et al. (2007) found only 38% percent mortality of *S. clava* after 40 h of air exposure treatments, but Hillock and Costello (2013) observed 100% mortality of the ascidian *S. clava* when it was exposed to sun at ambient temperatures (15–29 °C) after 24 h, and *Ciona* spp. also exhibited 100% mortality after 6–24 h of air exposure (Hopkins et al. 2016). Air exposure may thus be a practical and efficient method to *in situ* eliminate ascidian biofouling in mussel aquaculture. A simpler technique by Ramsay et al. (2008) proposed that an increased stocking density of mussels on mussel ropes (>250 mussels per foot) significantly reduced ascidian fouling, probably due to increased suspension feeding by the mussels. Moreover, biocontrol recruitment for fouling may also be an effective management method. Specifically, the presence of *Haliotis iris* (Gmelin, 1791) and *Cookia sulcata* (Gmelin, 1791) gastropods and sea urchins significantly reduced or even eliminated biofouling cover in different immersed surfaces, also inhibiting the establishment of new biofouling in these substrates (Atalah et al. 2014; Sterling et al. 2016).

Although increased temperatures have been shown to be successful against most fouling organisms (Fischer et al. 1984; Whitehouse et al. 1985), in most cases, thermal treatments were also detrimental against mussels, since relatively small increases in ambient water temperature cause significant mortalities in bivalves (Rajagopal et al. 1999, 2003; Masilamoni et al. 2002). Although the results of this study have shown a seasonal effect on ascidian biofouling on mussels' physiology, it cannot be concluded whether a specific season is most effective on ascidian biofouling decrease, since seasonality differentially affects the biomarkers examined herein. However, it has been shown that environmental conditions and/or biofouling activity degrade the collagenous threads, reducing the mechanical integrity of the byssal complex and the overall fitness of *M. edulis*. The latter effect is magnified during the elevated summer temperatures (Moeser and Carrington 2006). Nevertheless, the differential tolerance of bivalve species to temperature should be highlighted. For instance, *Mytilopsis leucophaeata* (Conrad, 1831) appears to be more tolerant to high temperature stress in comparison with other co-occurring species such as *M. edulis* and *Dreissena polymorpha* (Pallas, 1771) (Rajagopal et al. 2005). Moreover, no conclusion can be reached based on the non or sporadic presence of ascidian biofouling in S3 and S4, and the subsequent absence of the physiological indexes investigated in this study. Complex environmental conditions could be attributed for this observation. No literature exists regarding the effect of ascidian fouling intensity/density on mussel stress physiology. Since the present results exhibit an exceeded presence of ascidians covering more than 70% of the mussel sleeve surface in S1, S2 and S5, no conclusions can be drawn regarding this seasonal effect. Judging from the above,

laboratory experiments investigating the 'cause and effect' are necessary in order for the biofouling effect, and its intensity/density, to be enlightened in regards to the underlying physiological mechanisms, since these effects in nature are far more complex, and extreme weather events such as heat waves and large storms are likely to become more frequent or intense with human-induced climate change.

In conclusion, despite the increased seawater temperature stressing farmed *M. galloprovincialis* mussels, the presence of ascidians multiplied all examined stress biomarkers, providing clear evidence that ascidians constitute a major stressor of farmed bivalves. Taken all together, the presence of ascidians on aquaculture facilities and infrastructure, considered synergistically with climate change effects, such as seawater temperature increase, eutrophication and general rapid seasonal fluctuations of abiotic factors, may form a catastrophic combination for bivalves' health and molluscs farming. Therefore, although the current results evaluate for the first time the stress response of *M. galloprovincialis* under field conditions against this biotic factor, enlightening the biological impact of this phenomenon, additional research is needed in order to provide valuable information regarding the mechanisms underlying biofouling, and future studies should also evaluate the impact of ascidians and high temperature on the mussel physiology in the lab. The latter is rather important, as meta-analysis has revealed that biofouling typically reduces shellfish fitness and treatment options are often harsh, which could deleteriously affect stock (Sievers et al. 2017). Therefore, insights for future management of the marine aquatic sector could be provided and subsequently address measures for the decrease or elimination of this phenomenon and restoration of marine production.

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Data availability statement

Available after a reasonable request from the corresponding author

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